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Summary of Ph.D. Thesis

QUALITY BY DESIGN DRIVEN DEVELOPMENT OF POLYMERIC AND LIPID-BASED NANOCARRIERS AS POTENTIAL SYSTEMS FOR ORAL DELIVERY OF GLP-1 ANALOGUES

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- **PUBLICATIONS RELATED TO THE TOPIC OF THE THESIS**

I. **Ismail R**, Bocsik A, Katona G, Gróf I, Deli MA, Csóka I.
Encapsulation in polymeric nanoparticles enhances the enzymatic stability and the permeability of GLP1 analog- liraglutide across a culture model of intestinal permeability.
Pharmaceutics 11,599,2019.
(IF: 4.773), Q1

II. **Ismail R**, Sovány T, Gácsi A, Ambrus R, Katona G, Imre N, Csóka I.
Synthesis and statistical optimization of poly (lactic-co-glycolic acid) nanoparticles encapsulating GLP-1 analog designed for oral delivery.
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IV. **Ismail R** and Csóka I. Novel strategies in the oral delivery of antidiabetic peptide drugs – Insulin, GLP1 and its analogs.
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V. **Ismail R**, Phan TNQ, Laffleur F, Csóka I, Schnürch AB. Hydrophobic ion pairing of a GLP-1 analogue for incorporating into lipid nanocarriers designed for oral delivery.
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- **PUBLICATIONS NOT RELATED TO THE TOPIC OF THE THESIS**

I. Akel H**, Ismail R**, Csóka I. Progress and perspectives of brain-targeting lipid-based nanosystems via the nasal route in Alzheimer's disease.
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II. Sabir F, **Ismail R**, Csóka I. Nose to brain delivery of anti-glioblastoma drugs embedded into lipid nanocarrier system: status quo and outlook.
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- **PRESENTATIONS RELATED TO THE TOPIC OF THE THESIS**

Verbal presentation

I. **Ismail R** and Csóka I. Potential of polymeric and Lipid-based nanocarriers for oral GLP-1 analogue delivery. *II. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science*, Szeged, Hungary,2020.

II. **Ismail R** and Csóka I. Design of experiment DoE based methodology for designing polymeric NPs encapsulating Liraglutide for oral delivery. *12th Central European Symposium on Pharmaceutical Technology and Regulatory Affairs,* Szeged, Hungary,2018.

III. **Ismail R** and Csóka I. Quality by Design driven development of Liraglutide loaded nanocarrier system designed for oral delivery. *I. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science*, Szeged, Hungary,2019

IV. **Ismail R** and Csóka I. QbD based strategy for the development of oral antidiabetic peptide containing nanocarrier system. *1st Young Technologists' Forum*, Budapest, Hungary,2018.

I. **Ismail R** and Csóka I. Up to date advances in nano-carrier systems for oral delivery of antidiabetic peptides. *13th International Conference and Exhibition on Nanomedicine and Pharmaceutical Nanotechnology*, Rome, Italy,2017.

Poster presentation

I. **Ismail R** and Csóka I. In vitro appraisal of polymeric nanoparticles designed for oral delivery of GLP-1 analog- liraglutide. *12th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology,* Vienna, Austria,2020.

II. Csóka I, Katona G, Dorina D, **Ismail R**, Manteghi R, Pallagi E. Colloidal systems as carriers for peptide drugs: possibilities and challenges. *EUFEPS*, Frankfurt, Germany,2019.

III. Csóka I, Katona G, **Ismail R**, Pallagi E. Expectations and limits of colloidal systems as carriers for peptide drugs. *3rd International Symposium on Scientific and Regulatory Advance in Biological and Non-Biological Complex Drugs: A to Z in Bioequivalence*, Budapest, Hungary,2018.

IV. **Ismail R** and Csóka I. Pharmaceutical applications of colloidal drug delivery systems: case studies for biological drugs. *11th Conference on Colloid Chemistry*, Eger, Hungary,2018.

V. **Ismail R** and Csóka I. Initial SWOT analysis of designing polymeric nanoparticles and lipid-based nanoparticles for oral GLP-1 analog delivery. *9th International Congress Nanotechnology in Biology and Medicine*, Graz, Austria,2018.

VI. **Ismail R** and Csóka I. Quality by design (QbD) oriented study for designing oral Liraglutide loaded polymeric nanoparticles. *11th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology*, Granada, Spain,2018.

VII. **Ismail R** and Csóka I. Evaluation of techniques and possibilities for oral delivery of antidiabetic peptide drug -liraglutide- in a novel delivery system. *7th BBBB International Conference on Pharmaceutical Sciences*, Budapest, Hungary,2017.

1. INTRODUCTION AND AIMS

Type 2 diabetes mellitus (T2DM) is a progressive disorder characterized by the production of insufficient level of insulin due to β cell dysfunction and/or insulin resistance, accounting for roughly 90% of all diabetic cases (American Diabetes Association, 2015). With the expanding knowledge of the pathophysiology of T2DM, the incretin system has become a crucial target in the treatment of T2DM patients. There are two approved classes of incretin-based therapeutics: glucagon-like peptide-1 (GLP-1) analogues and dipeptidyl peptidase-4 inhibitors (DPP-4i). Glucagon-like peptide-1 analogues liraglutide (Lira) and exenatide (Exn) are currently limited to subcutaneous injection (SC) in clinical protocols. Due to several drawbacks accompanied by this invasive route, the development of a patient-friendly delivery system should be aimed for (Araújo *et al.*, 2012). Herein, the oral route is likely the most desirable choice since it mimics the physiological GLP-1 secretion in addition to ensuring good patient adherence to the treatment (Lin *et al.*, 2016).

Abbreviations:

ADA – American Diabetes Association; ANOVA – Analysis of Variance; AUC – Area Under Curve; BAR – Relative Bioavailability; CMAs – Critical Material Attributes; Cmax – Maximum Serum Concentration; CPPs – Critical Process Parameters; CQAs – Critical Quality Attributes ;DS – Design Space; DOC – Sodium Docusate; DoE – Design of Experiment; DPP-4i – Dipeptidyl Peptidase-4 Inhibitors; EE – Encapsulation Efficiency; ELISA – Enzyme-Linked Immunosorbent Assay; EMA – European Medicine Agency ; EASD – European Association for the Study of Diabetes; Exn – Exenatide; GI – Gastrointestinal ; GLP – 1-Glucagon-Like Peptide-1; HPLC – High performance Liquid Chromatography; I.V. – Intravenous; ICH – International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use; Lira – Liraglutide; log D SEDDS/release medium – The distribution coefficient of the drug/ion pairs between the oily droplet phase of SEDDS and the release medium; MEM – Minimum Essential Medium Eagle; MS – Mean Square; NCs – Nanocarriers; NPs – Nanoparticles; O/W – Oil on Water; P.O. – Per Oral; P_{app} *– Apparent Permeability; PBD – Plackett-Burman Design; PDI – Polydispersity Index; PG – Propylene Glycol; PLGA – Poly (D, L -Lactic-Co-Glycolic Acid); PVA – Poly (vinyl alcohol); QbD – Quality by Design; QTPP – Quality Target Product Profile; RA – Risk Assessment; SC – Subcutaneous; SD – Standard deviation; SEDDS- – Self-emulsifying Drug Delivery Systems; SGF – Simulated Gastric Fluid; SGFsp- – Simulated Gastric Fluid without enzymes; SIF – Simulated Intestinal Fluid; SIFsp – Simulated Intestinal Fluid without enzymes; T2DM – Type 2 Diabetes Mellitus; TEER – Transepithelial electrical resistance; TFA – Trifluoroacetic acid; THA – Tetraheptylammonium Bromide; Tmax – Time to reach maximum serum concentration; TX-100 – Triton X-100; USFDA – US Food and Drug Administration; W/O – Water to Oil ratio; W1/O/W2 – Water in Oil in Water.*

Moreover, oral delivery appears to be feasible for such antidiabetic peptides due to the relatively larger safety window of GLP-1 analogues compared to insulin. Among the various strategies having been developed to conquer the barriers limiting oral peptide delivery, namely the harsh environment of the gastrointestinal (GI) tract and the absorption membrane barrier, the encapsulation of GLP-1 analogues into nanosystems seems to be very promising strategy (Ismail and Csóka, 2017).

Therefore, the aim of this work was to investigate the potential of polymeric and lipid-based nanocarriers (NCs) in overcoming the main challenges which face a successful oral delivery of Lira and Exn. Owing to the complexity, biocompatibility and nanotoxicological concerns of nanopharmaceuticals, not to mention the risks accompanied with peptide drugs formulation development, it was crucial to implement the Quality by Design (QbD) and risk assessment (RA) concepts to develop a thorough understanding of the target product and process design (Pallagi *et al.*, 2018). Following to setting up the Quality Target Product Profile (QTPP) and conducting the initial RA for designing oral GLP-1 analogues loaded nanocarriers (NCs), two different NCs were designed, optimized and assessed according to their evaluated regarding the critical quality attributes (CQAs). These developed NCs are (i) Lira loaded poly (d, l-lactic-co-glycolic acid) nanoparticles (PLGA NPs) and (ii) Exn loaded self-emulsifying drug delivery system (SEDDS). Figure 1 presents the main experimental steps being followed in this work.

Extended QbD model - Risk assessment

- Preformulation design followed by setting up the QTPP for oral GLP-1 analogues loaded NCs.
- Defining the CQAs, CPPs and CMAs.
- Conducting the initial RA based study (Lean-QbD® software).

Design and evaluation of liraglutide loaded PLGA NPs

- Optimization of particle size, polydispersity index (PDI), surface charge and encapsulation efficiency (EE) by applying 7-factor, 2-level Plackett-Burman Design (Zetasizer. HPLC, Statistica® software).
- Assessment of formulation stability: particle size, PDI and zeta potential (Zetasizer).
- Evaluation of in vitro release characteristics (HPLC).
- Conducting enzymatic stability study (HPLC).
- Assessment of cytotoxicity (RTCA SP) and intestinal permeability across Caco-2 cell model (HPLC).

Design and evaluation of exenatide loaded SEDDS

- Optimization of hydrophobic ion pairs (HIPs) of Exn with cationic/anionic surfactants, and evaluating the precipitation efficiency and zeta potential of the HIPs (HPLC, Zetasizer).
- Development of SEDDS loaded with HIPs (exenatide:surfactant), and evaluation of particle size, PDI, zeta potential, and HIPs payload (Zetasizer, HPLC).
- Assessment of formulation stability: particle size, PDI and zeta potential (Zetasizer).
- Evaluation of in vitro release characteristics (determination of log D SEDDS/release medium of HIPs) (HPLC).
- Assessment of cytotoxicity (hemolysis assay) and intestinal permeability on freshly excised rat intestinal mucosa (ELISA, Microplate reader).
- In vivo pharmacokinetic study following the oral administration in healthy rats (ELISA, Microplate reader).

Figure 1. The main questions/tasks of this work to be answered.

2. MATERIALS

Liraglutide was purchased from Xi'an Health Biochem Technology Co., Ltd (Shaanxi, China). Exenatide was purchased from Chemos GmbH (Altdorf, Germany).

For PLGA NPs preparation, Poly (D,L-lactide-co-glycolide) (PLGA 50:50, Mw=30,000-60,000 Da), poly (vinyl alcohol) (MOWIOL 4-98®, MW~27000 Da), and D-(+)-Trehalose dihydrate (MW=378.33 g/mol) were purchased from Sigma-Aldrich (Munich, Germany). D- (-) -Mannitol was supplied from Molar Chemicals Ltd. (Budapest, Hungary). Sodium acetate anhydrous was supplied from Scharlau Chemie S.A. (Barcelona, Spain). Ethyl acetate used for dissolving PLGA was obtained from Reanal Labor (Budapest, Hungary). Pepsin from porcine gastric mucosa, powder (≥400 units/mg protein) and pancreatin from porcine pancreas (≥3× USP specifications) were purchased from Sigma Aldrich (Budapest, Hungary). The Caco-2 intestinal epithelial cell line was purchased from ATCC (cat.no. HTB-37) at passage 60. Dulbecco's modified Eagle's medium (Gibco, Life Technologies, Carlsbad, CA, USA) and 10% fetal bovine serum (Pan-Biotech GmbH, Aidenbach, Germany) were used as cell culture supplements.

For SEDDS preparation, Capmul® MCM (mono/diglycerides of caprylic acid, HLB 5-6) and Captex® 355 (caprylic/capric triglycerides, HLB 0) were supplied from Abitec (Janesville, Wisconsin, USA). Kolliphor® RH 40 (polyoxyl hydrogenated 40 castor oil, HLB 14-16), sodium docusate and tetraheptylammonium bromide were purchased from Sigma-Aldrich (Vienna, Austria). Minimum Essential Medium Eagle (MEM) and Dulbecco's phosphate-buffered saline were supplied from Biochrom GmbH (Berlin, Germany). Exendin-4 fluorescent ELISA kit was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). Hanks' Balanced Salts (HBSS), propylene glycol and all other reagents and solvents were purchased from Sigma-Aldrich (Vienna, Austria).

3. METHODS

3.1. QbD based strategy for development of GLP-1 analogue loaded nanocarriers

At the preformulation design stage, all the possibilities concerning the administration route, dosage form and drug substance were carefully evaluated. This was followed by the identification of the quality target product profile (QTPP) of GLP-1 analogue loaded nanocarrier. Defining the CQAs, CMAs and CPPs was the next step, followed by performing the initial RA with Lean QbD® Software (QbD Works LLC. USA, CA, Fremont).

3.2.Liraglutide loaded PLGA NPs

3.2.1. Preparation of liraglutide loaded PLGA NPs

The preparation of Lira loaded PLGA NPs was carried out by means of the double emulsion W1/O/W2-solvent evaporation method (Ismail *et al*., 2019b). The nanoparticles were lyophilized at -40 °C at 0.01 mbar (Scanvac, CoolSafe 100-9 Pro freeze dryer). A 5-10% (m/v) of mannitol or trehalose was added as lyoprotectants.

3.2.2. Optimization of liraglutide loaded PLGA NPs: Plackett-Burman design (PBD)

PBD with a total of 8 runs involving 7 independent variables at two levels was carried out using STATISTICA 13® software. Particle size (Y1), polydispersity index (PDI) (Y2), encapsulation efficiency (EE) (Y3) and zeta potential (Y4) were selected as dependent variables. The design was validated, and the optimized formulation was prepared within the DS and compared with the predicted values of the responses.

3.2.3. Characterization of the developed liraglutide loaded PLGA NPs

3.2.3.1. Particle size, size distribution, surface charge and encapsulation efficiency measurements

The hydrodynamic diameter (Z-average), PDI and zeta potential of NPs reconstituted in demineralized water were measured in a folded capillary cell by using Malvern Nano ZS Zetasizer (Malvern Instruments Ltd. UK) equipped with He-Ne laser (633 nm). The EE of Lira loaded in PLGA NPs was determined directly using the centrifugation method (Ismail *et al.*, 2019b).

3.2.3.2. In vitro release study

The release behavior of Lira from PLGA NPs was evaluated in simulated gastric fluid without enzymes (SGFsp: 0.1N HCl at pH 1.2) over 2 hours followed by simulated intestinal fluid without enzymes (SIFsp: phosphate buffer saline at pH 6.8) over 4 hours. Samples were centrifuged at 16,500 ×g and 4°C for 10 min, and Lira concentration in the supernatant was determined by HPLC.

3.2.3.3. Enzymatic degradation study

Lira loaded PLGA NPs were resuspended in pepsin containing SGF or pancreatin containing SIF and incubated at 37°C under stirring of 100 rpm. Native Lira was used as control. Samples were withdrawn at specific time intervals over a 2-hour period, and an equal volume of ice-cold reagent was added: 0.1 M NaOH for SGF and

0.1 M HCl for SIF, to stop the enzymatic reaction. Samples were centrifuged at 16,500 ×g and 4°C for 10 min and the supernatant was analyzed by HPLC.

3.2.3.4. Treatment of Caco-2 cells

The Caco-2 cells were grown, as previously reported (Bocsik *et al.*, 2019). The concentration of stock solutions for cell culture experiments was 1 mM for both the therapeutic peptide Lira and the PN159 peptide, which was used as a reference absorption enhancer.

3.2.3.5. Cell viability measurement by impedance

Impedance was measured at 10 kHz by an RTCA SP instrument. Lira, Lira-loaded/free PLGA NPs, Lira and PN159 solution, and PN159 peptide were diluted in cell culture medium and the effects were followed for 24 h. Triton X-100 (TX-100) (1 mg/ml) was used as a reference positive control.

3.2.3.6. Permeability study on the Caco-2 cell model

Transepithelial electrical resistance (TEER) monitoring was performed by an EVOM volt-ohmmeter (World Precision Instruments, Sarasota, FL, USA) combined with STX-2 electrodes. In the donor (upper/apical) compartments, the culture medium was replaced by 0.5 mL Ringer-buffer containing treatment solutions of Lira, Lira loaded in PLGA NPs, and Lira and PN159 solution at the concentration of 100 µM for Lira for 1 h. Treatment solutions from both compartments were collected and the Lira level was detected by HPLC. The apparent permeability coefficients (P_{app}) and recovery were calculated as described previously (Hellinger *et al.*, 2012; Bocsik *et al.*, 2016).

3.2.3.7. Immunohistochemistry

Aiming to investigate the morphological changes in interepithelial junctions, immunostaining for the junctional proteins, zonula occludens protein-1 (ZO-1) and β-catenin, was carried out (Ismail *et al*., 2019a). Hoechst dye 33342 was used to stain the cell nuclei. After mounting the samples, the staining was visualized by a Visitron spinning disk confocal system (Visitron Systems GmbH, Puchheim, Germany).

3.3.Exenatide loaded SEDDS

3.3.1. Optimization of hydrophobic ion-pairing of exenatide with THA and DOC

Exn acetate aqueous solution was prepared in a concentration of 2 mg/ml and the pH was adjusted with 0.1 M NaOH to 8.0. Increasing amounts of THA were dissolved in 1 ml of demineralized water: methanol and added slowly and dropwise

to 1 ml of the Exn solution under light shaking. Exn -DOC ion pairs were prepared as previously described (Menzel *et al.*, 2018), where the pH of 2 mg/ml Exn solution was adjusted to 3.0 with 2 M HCl followed by the addition of an equal volume of DOC aqueous solution slowly and dropwise to Exn solution under light shaking.

The supernatant that contained the remaining amount of dissolved Exn was analyzed by HLPC to calculate the precipitation efficiency. Zeta potential of HIPs with surfactant was determined with Zetasizer Nano ZSP.

3.3.2. Development of Exn-THA and Exn-DOC loaded SEDDS

SEDDS were formed by dissolving 2 mg of HIPs with THA or DOC having been prepared as described above in 4 mg of PG, 41 mg of Capmul MCM, 40 mg of Kolliphor RH and 15 mg of Captex 355 and homogenizing the mixture (1500 rpm) after the addition of each component.

3.3.2.1. Particle size, size distribution, surface charge and payload measurements

To determine the maximum payload of HIPs of Exn-surfactant in the formulation, the preconcentrate was analyzed by HPLC. The droplet size (Z-average), PDI and zeta potential of SEDDS were measured via Zetasizer Nano ZSP.

3.3.2.2. Determination of log D $_{SEDDS/release\ medium}$ of Exn-THA and Exn-DOC

Lyophilized Exn-THA (molar ratio 1:8) and Exn-DOC (molar ratio 1:4) were dispersed in SIF and HBSS. After stirring for 3 hours at 1000 rpm, the suspension was centrifuged for 5 min at 13400 rpm. The supernatant was analyzed by HPLC, and log $D_{SEDDS/RM}$ of HIPs was calculated.

3.3.2.3. In vitro hemolysis assay

In-vitro hemolysis assay of Exn-THA SEDDS, Exn-DOC SEDDS and blank SEDDS was carried out according to a previously reported protocol (Lam *et al.*, 2019). The percentage of hemolysis (H%) was calculated.

3.3.2.4. Ex-*vivo* permeability study

The ex-vivo permeability study was performed on fasted rats weighing 200-250 g. Rats were sacrificed and the freshly excised small intestine was preincubated in HBSS buffer for 30 min before being cut into strips of 2 cm mounted in Ussing-type chambers. The fresh samples at the apical side were: (1) 0.25% (m/v) of Exn-THA-SEDDS, (2) 0.6% (m/v) of Exn-DOC SEDDS and (3) 8 µg /ml of Exn solution in HBSS at the apical side. Exn concentration in the aliquots withdrawn from the

basolateral side over 3 hours was quantified by fluorescent ELISA immunoassay (FEK-070-94; Phoenix Pharmaceuticals, Inc., USA). The absorbance was read at 450 nm using a microplate reader (Spark Multimode microplate reader, Tecan).

3.3.2.5. In-*vivo* study

Male Sprague-Dawley rats with a mean bodyweight of 200-250 g were supplied by Janvier Labs (Saint Berthevin, France) and the in *vivo* study was approved by the Ethical Committee of Austria and performed according to the principles of Laboratory Animal Care. Rats were randomly divided into four groups (n = 5). The first group was the positive control group where Exn solution was administered via SC injection at a dose of 50 µg/kg body weight. The second group was the negative control group where Exn solution was orally administered at a dose of 300 µg/kg body. The third and fourth group received 300 µg/kg body weight of Exn-THA and Exn-DOC SEDDS formulation via oral gavage, respectively.

The serum was separated from the collected blood samples and stored at -80 ^{o}C for further analysis. Exn concentration was measured by fluorescent ELISA immunoassay. The absorbance was read at 450 nm using a microplate reader. The relative bioavailability (BA_R) of Exn after oral administration was calculated.

4. RESULTS and DISCUSSION

4.1.QbD based strategy for development of GLP-1 analogue loaded nanocarriers

The initial step of QbD-based study aiming to design oral GLP-1 analogue loaded nanocarriers was to set up the QTPP based on the relevant guideline (USFDA, 2009; 2017). The proposed CQAs, CMAs and CPPs regarding the preparation methods of PLGA NPs and SEDDS were also defined. Ishikawa fishbone diagram was established to configure the risk analysis process for defining the cause-effect relationship between the significant variables and the CQAs of the NCs (PLGA NPs, SEDDS) (Figure 2).

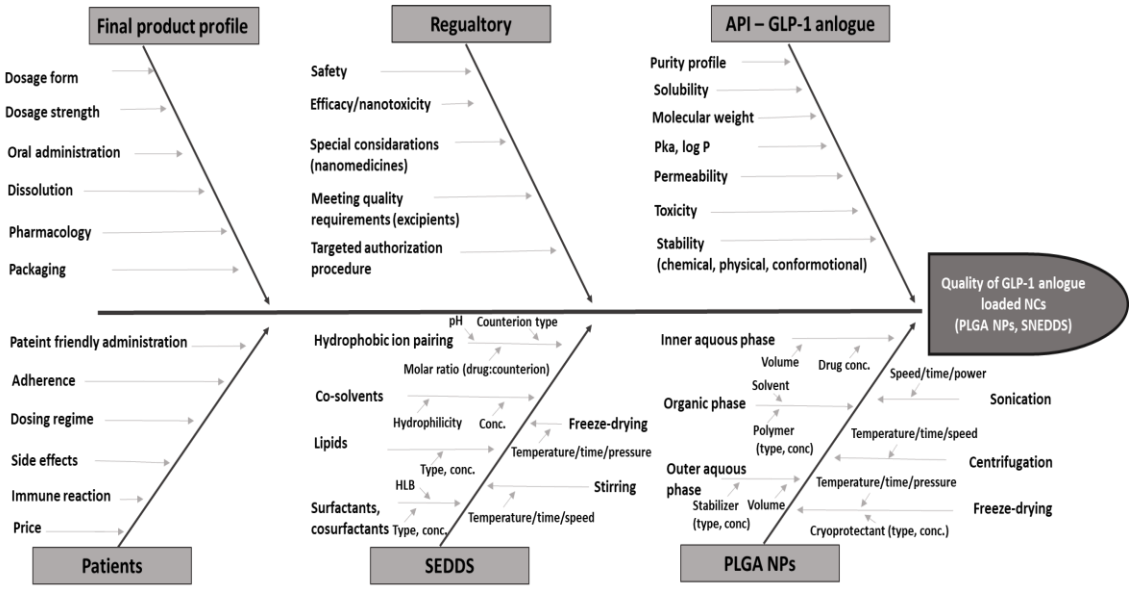

Figure 2. Ishikawa fishbone diagram for evaluating the risky factors related to the quality of GLP-1 analogue loaded into nanocarriers (NCs).

Results of the RA study revealed that in the case of PLGA NPs, the most highly influential CPP is sonication time, and the most highly influential CMAs are polymer concentration, drug concentration, stabilizer concentration, cryoprotectant type, cryoprotectant concentration and external aqueous phase to organic phase ratio W2/O. Regarding SEDDS, the most highly influential CPP is the pH level during the HIPs formation, and the highly influential CMAs are also related to HIPs formation (type of counterion, molar ratio of peptide to counterion) followed by the surfactant type and concentration.

4.2.Liraglutide loaded PLGA NPs

4.2.1. Optimization of liraglutide loaded PLGA NPs: Plackett-Burman design (PBD)

The high risk formulation and process parameters resulted from RA study were further investigated regarding their effects on four responses namely: particle size (Y1), PDI (Y2), EE (Y3) and zeta potential (Y4) by applying a seven-factor, two-level, eight-run PBD (Table 1).

Table 1. Results of experimental responses in Plackett-Burman Design (PBD). Data are presented as mean ± SD (n=3).

Run code	Particle size (Z-Average) (nm)	PDI	EE%	Z-potential (mV)
PBD-F1	160.11±5.63	0.1±0.03	20.07±1.7	-30.57±1.79
PBD-F2	209.82±8.01	0.15±0.01	36.01±1.25	-24.97±1.64
PBD-F3	190.01±2.81	0.23±0.01	40.98±2.46	-27.35±0.67
PBD-F4	200.21±3.47	0.16±0.03	43.47±3.34	-31.15±1.23
PBD-F5	179.47±3.83	0.17±0.01	31.91±4.03	-23.83±0.95
PBD-F6	235.69±5.34	0.09±0.003	28.83±2.05	-29.24±0.41
PBD-F7	223.59±3.74	0.17±0.02	22.17±2.12	-30.42±0.39
PBD-F8	183.52±3.03	0.2±0.01	20.96±1.51	-26.95±0.148

After establishing the polynomial equations, particle size and EE were found to be significantly affected by almost all the tested CPPs and CMAs. The minor differences between the predicted values and the average of experimental values confirm the validity of this design in providing a good prediction of the four tested responses (Table 2).

Table 2. The observed and the predicted values of the response values of the optimized Liraglutide loaded PLGA nanoparticles. Data are presented as mean ± SD (n=3).

Experimental response	Predicted value	Observed value (F1) (Bias%)	Observed value (F2) (Bias%)	Observed value (F3) (Bias%)
Particle size (nm)	197.8	195.8±2.5 (1.0)	202.72±5.8 (2.5)	196.41±4.06 (0.7)
EE (%)	30.5	29.46±1.83(3.3)	31.18±2.32 (2.3)	33.14±3.32 (8.5)
PDI	0.2	0.19±0.01 (5.5)	0.19±0.01(5.0)	0.18±0.003 (10.0)
Zeta potential (mV)	-28.1	-28.11±1.06 (0)	-28.84±2.51 (2.5)	-27.7±1.33 (1.4)

The DS was optimized targeting the following criteria: the particle size was minimized; EE was maximized while PDI and Zeta potential were excluded. Thanks to the knowledge obtained via the DS, the optimum levels of the formulation factors were determined: 60 mg of PLGA, 5 mg of Lira, 0.5 min 2nd sonication time, 1.48% of PVA, 5% of mannitol and W2/O ratio of 5. The optimized formula showed a particles size of 188.95±4.99 nm and EE of 51.81±2.93%.

4.2.2. Drug release study

The release behavior showed a biphasic release pattern starting by a moderate initial burst release during the first 2 hours in SGFsp, where 14.2±0.86% of Lira was released from the NPs. This was followed by a slow release profile until 6 hours in the SIFsp (Figure 3A).

4.2.3. Enzymatic degradation study

The encapsulation of Lira into PLGA NPs was able to successfully protect 71.2±1.49% and 87.6±1.3% of Lira from degradation in the SGF and SIF at the end of 2-hour incubation, respectively (Figure 3B). These results depict that PLGA NPs can provide a physical barrier between the encapsulated Lira and the hostile environment of GI tract.

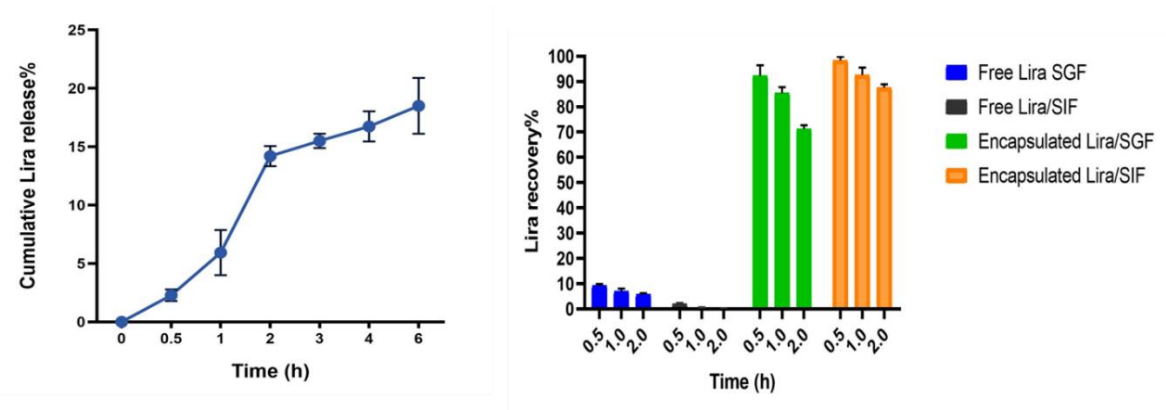

Figure 3. **(A)** Cumulative in vitro release profile of liraglutide (Lira) from PLGA NPs. **(B)** Enzymatic stability of Lira encapsulated in PLGA NPs in SGF and SIF media, with free Lira as a control. SGF, Simulated Gastric Fluid; SIF, Simulated Intestinal Fluid.

4.2.4. Cell viability measurement by impedance

As illustrated in Figure 4, the impedance measurement did not exhibit any significant cell damage after treatments with Lira, Lira loaded in PLGA NPs, Lira with PN159 peptide, unloaded PLGA NPs or PN159 peptide. Figure 4A shows the kinetics of the cellular effects of treatment solutions, while the columns on Figure 4B show the effect of treatments at the 1-hour time point.

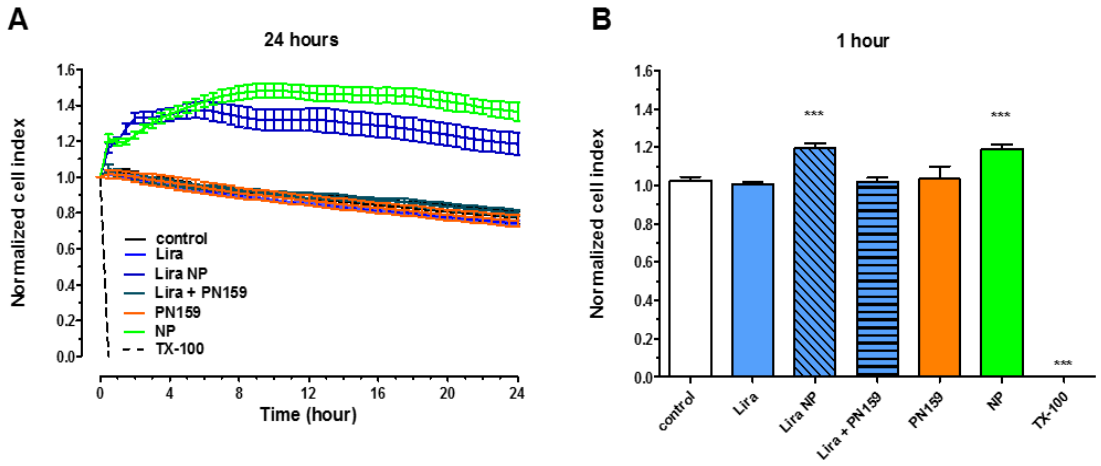

Figure 4. (A) Cell viability kinetics for 24 hours and **(B)** results of a 1-hour treatment of Caco-2 intestinal epithelial cells with liraglutide (Lira), Lira in PLGA NPs, Lira with PN159 peptide, blank PLGA NPs and PN159 peptide measured by impedance. Values are presented as means ± SD, n = 6–12. Statistical analysis: Analysis of Variance (ANOVA) followed by Dunnett's test. NPs, nanoparticles; TX-100, Triton X-100. ***p<0.001 compared to control.

4.2.5. Permeability study on the Caco-2 cell model

Lira encapsulated in PLGA NPs showed a 1.5-fold higher apparent permeability as compared to Lira alone (Figure 5A). There was no statistical difference between Lira loaded PLGA NPs and Lira + PN159 groups in enhancing the permeability across Caco-2 cells. On the contrary of group containing PN159 peptide where the TEER values dropped after the 1-hour treatment (Figure 5A), Lira alone or loaded in PLGA NPs did not change the ionic permeability (Figure 5B), suggesting no toxic effect on differentiated Caco-2 cells and no effect on the paracellular pathway.

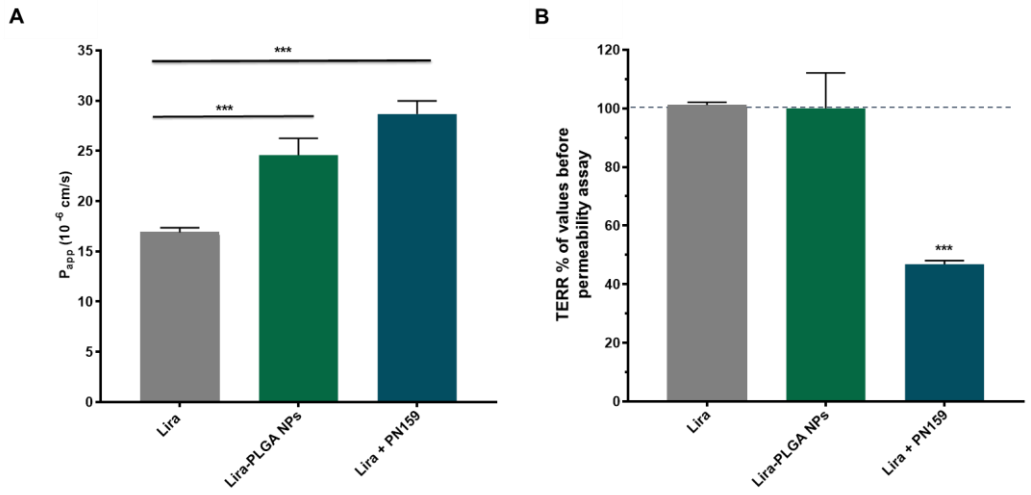

Figure 5. (A) Evaluation of permeability of liraglutide (Lira) (100 µM) across Caco-2 epithelial cell layers treated with different Lira formulations for 1 h. **(B)** Changes in transepithelial electrical resistance (TEER) values of Caco-2 cell layers after 1-hour treatment with different Lira formulations as compared to TEER values before treatment. Data are presented as means ± SD (n = 4). Statistical analysis: ANOVA followed by Bonferroni test, ***p<0.001 compared to Lira group.

4.2.6. Immunohistochemistry

No morphological changes of interepithelial junctions were observed with Lira-PLGA NPs treated group confirming the lack of a paracellular component in the transport mechanism (Figure 6). Only the PN159 peptide treated group demonstrated a visible change in the staining pattern of β-catenin adherens junctional protein.

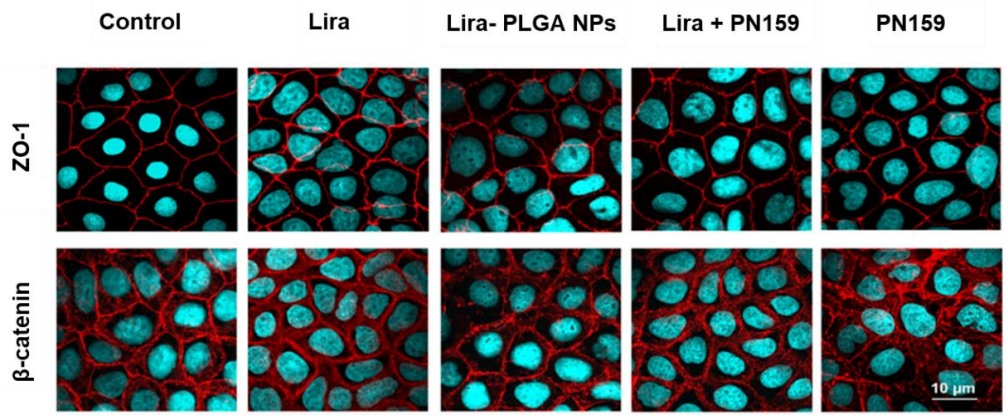

Figure 6. Effects of Lira, Lira-PLGA NPs, Lira and PN159 peptide, and PN159 peptide on the junctional morphology of Caco-2 epithelial cells. Immunostaining for zonula occludens-1 (ZO-1), and β-catenin junction proteins after a 1-hour treatment. Red color: immunostaining for junctional proteins. Blue color: staining of cell nuclei. Bar: 10 µm.

4.3.Exenatide loaded SEDDS

4.3.1. Optimization of hydrophobic ion-pairing (HIP) of exenatide with (THA)

Different Exn to surfactant ratios were evaluated as illustrated in Figure 7. The more surfactant was added to Exn, the more HIPs were formed until the maximum precipitation efficiency of 95.3 ± 4.02% at molar ratio of 1:8 (Exn: THA), whereas a maximum of 100% was reached in the case of DOC at molar ratio of 1:4 (Exn: DOC). Higher concentrations of surfactant led to a lower amount of formed ion pairs.

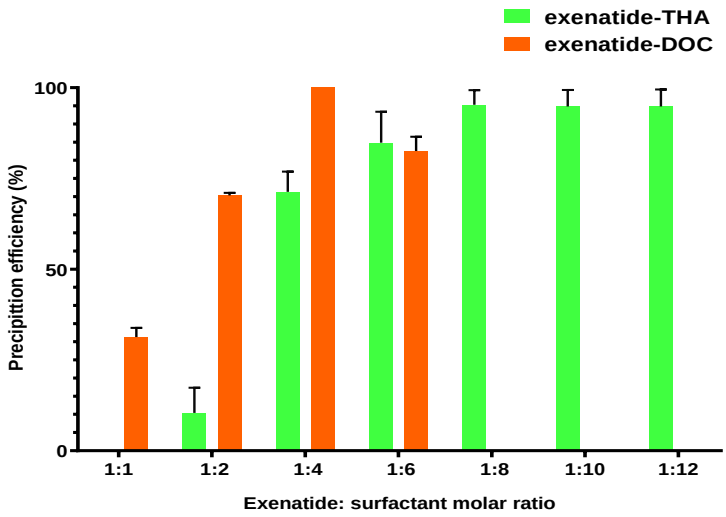

Figure 7. Precipitation efficiency of exenatide (2 mg/mL) with THA and DOC at different molar ratios. The precipitated exenatide-surfactant ion pairs were centrifuged and the remaining amount of exenatide in supernatant was quantified by HPLC. Data are presented as mean ± SD (n = 3).

The shift in zeta potential of HIPs having been formed at different molar ratios of Exn to surfactant was also determined in order to evaluate the surface charge of the complexes (Figure 8).

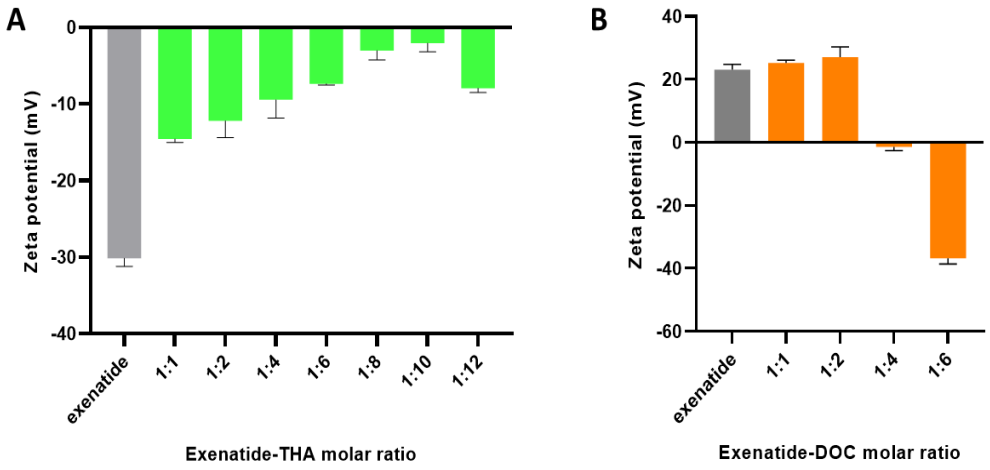

Figure 8. The shift in zeta potential values of **(A)** exenatide-THA HIPs formed at pH 8.0 in different molar ratios, and **(B)** exenatide-DOC HIPs formed at pH 3.0 in different molar ratios. Data are presented as mean ± SD (n = 3).

4.3.2. Characterization of exenatide-THA and exenatide-DOC loaded SEDDS

4.3.2.1. Particle size, size distribution, surface charge and payload measurements

Small droplet size (< 30 nm) with low PDI value was determined for loaded SEDDS. The surface charge of Exn-THA loaded SEDDS was positive whereas negative values were measured in case of Exn-DOC loaded SEDDS. This observation can be explained by the cationic character of THA and the anionic character of DOC. The maximum payload of Exn-THA and Exn-DOC that could be dissolved in the pre-concentrate was 0.54% and 0.17%, respectively.

4.3.2.2. Determination of log D $_{SEDDS/release\ medium}$ of exenatide-THA and exenatide-DOC

A log D of at least 2 was required to keep ≥ 50% of HIPs in SEDDS. The log $D_{SEDDS/RM}$ of Exn-THA was 2.29 and 1.92, whereas the log $D_{SEDDS/RM}$ of Exn-DOC was 1.2 and -0.9 in simulated intestinal fluid and HBSS, respectively. These log D values proved that THA is capable of forming more hydrophobic complexes than DOC.

4.3.2.3. Cytocompatibility

Both blank and loaded SEDDS displayed no significant hemolytic activity at concentrations of 0.1% (m/v) and 0.25% (m/v) (Figure 9). Compared to SEDDS loaded with Exn-THA, SEDDS loaded with Exn-DOC showed higher toxicity as concluded from the hemolytic activity reaching more than 90% at a concentration of 0.5% (m/v) of this formulation.

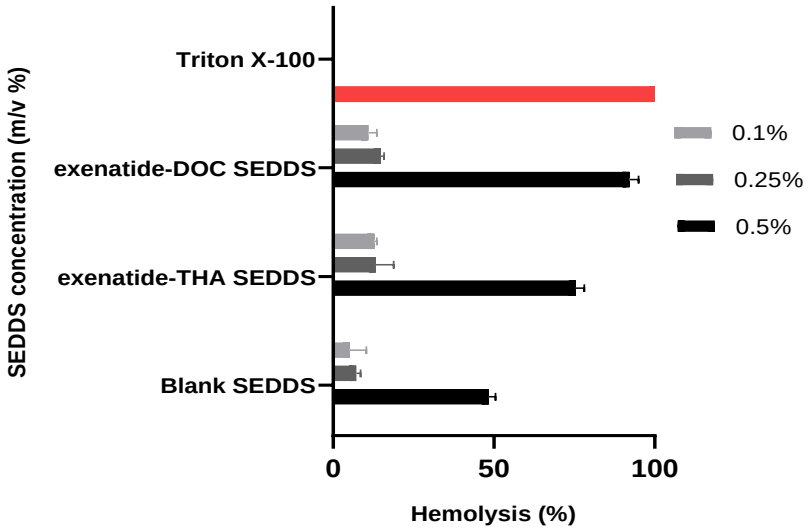

Figure 9. Cytotoxicity of different concentrations of blank SEDDS, exenatide-THA SEDDS, exenatide-DOC SEDDS. Hemolysis assay was conducted with human red blood cells (RBC). Data are presented as mean ± SD (n = 3).

4.3.2.4. Ex-vivo permeability study

Results presented in Figure 10 clearly demonstrate that Exn-DOC SEDDS and Exn-THA SEDDS yielded a 3-fold and a 10-fold enhancement of intestinal membrane permeability compared to free Exn. This outcome provides evidence for the crucial role of the type of HIPs incorporated in SEDDS in enhancing permeability across the intestinal mucus gel layer and epithelial barrier strongly limiting oral peptide uptake.

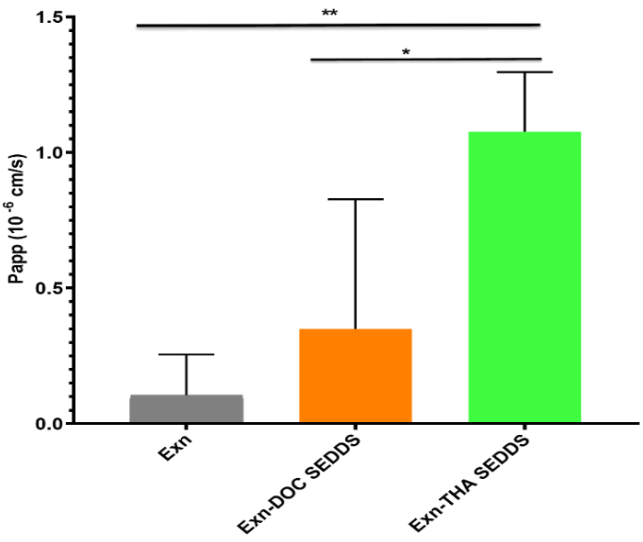

Figure 10. Evaluation of ex vivo permeability of exenatide (Exn) across rat intestinal mucosa following the 3-hour treatment with free Exn solution, Exn-THA SEDDS or Exn-DOC SEDDS. Values are presented as means ± SD (n = 3). Statistical analysis: Analysis of Variance (ANOVA) followed by Bonferroni test, * $p < 0.05$, ** $P < 0.01$.

4.3.2.5. In-vivo study

As illustrated in Figure 11, SC injection of free Exn solution elicited a maximum serum level at 1 hour after administration while the oral administration of Exn-THA and Exn-DOC SEDDS elicited a maximum serum level after 3 hours.

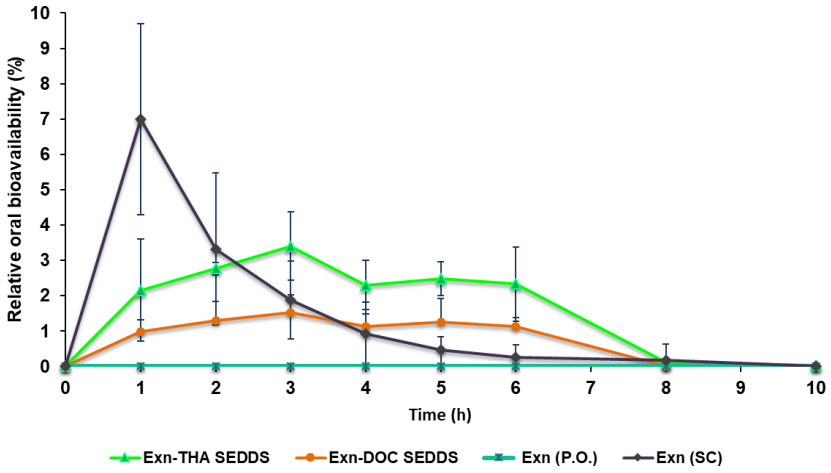

Figure 11. Exenatide (Exn) plasma concentration vs time profiles for SC free exenatide solution (50 µg/kg), oral exenatide solution (dose: 300 µg/kg), oral exenatide-THA SEDDS (dose: 300 µg/kg) and oral exenatide-DOC SEDDS (dose: 300 µg/kg). Values are presented as means ± SD (n = 5).

The developed SEDDS incorporating Exn-DOC resulted in a greater BA_R (16.29±5.34%) when administered to rats (Table 3), compared to the previously reported formulation (Menzel *et al.*, 2018). Exn-THA SEDDS, however, showed an even greater potential reaching a BA_R of 27.96 ± 5.24% (Table 3), which is in consistence with ex-*vivo* results confirming the superiority of this system compared to Exn-DOC SEDDS.

Table 3. Pharmacokinetic parameters of exenatide in rats following subcutaneous (SC) injection of the free exenatide solution and per oral (P.O.) administration of exenatide solution, exenatide-THA SEDDS and exenatide-DOC SEDDS. C_{max}: maximum serum concentration; T_{max}: time at which C_{max} is reached; AUC 0-10: area under the serum concentration-time curve over 10 h, BA_R: relative bioavailability (n = 5).

Formulation	**Free exenatide solution**	**Exenatide-THA SEDDS**	**Exenatide-DOC SEDDS**
Route of administration	SC	P.O.	P.O.
Dose (µg/Kg)	50	300	300
Cmax (ng/ml)	7.0±2.70	3.4±0.97	1.53±0.50
Tmax (h)	1	3	3
AUC 0-10 (ng h/ml)	10.56±3.92	17.33±3.48	10.28±4.7
BA_R (%)	100	27.96±5.24	16.29±6.63

5. SUMMARY

Since the oral delivery of GLP-1 analogues Lira and Exn can improve the patient adherence to therapy, a smart carrier system that can tackle the challenges hindering the oral delivery of these peptides has been targeted. In accordance with our research goals, polymeric and lipid-based nanocarriers were designed for the oral delivery of Liraglutide and Exenatide. The extended QbD model-based development of GLP-1 analogues loaded nanocarriers was successfully implemented to identify and rank the potentially high-risk attributes.

PLGA NPs encapsulating Lira were prepared by double emulsion solvent evaporation method. A seven-factor, two-level, eight-run Plackett-Burman DoE was a validated and useful statistical tool to be applied for the optimization of PLGA NPs

containing Lira. The optimized formulation was prepared within the defined Design Space. Lira loaded PLGA NPs with a homogeneous distribution, particle size of 188.95 nm and encapsulation efficiency of 51.81% which were within the desired range. The physical stability of this formulation was also proved over one week. The PLGA nanosystem could hinder the release of loaded Lira in stomach and then release it in the small intestine for absorption. The ability of PLGA NPs to protect the encapsulated Lira from hostile environment of GI tract was further confirmed by enzymatic stability studies. The biocompatibility of free/loaded PLGA NPs was proved in Caco-2 cells. Compared to free Lira, Lira encapsulated in PLGA NPs showed a 1.5-fold enhancement in intestinal apparent permeability which was comparable to Lira + PN159 group. On the contrary of PN159 containing group, no morphological changes of interepithelial junctions ZO-1 and β-catenin were observed with Lira loaded PLGA NPs treated group confirming the lack of a paracellular component in the transport mechanism.

HIPs of Exn with THA (cationic surfactant) and DOC (anionic surfactant) were formed before being incorporated into SEDDS. The maximum ion-pairing efficiency was reached after optimizing the molar ratio (Exn: surfactant) and pH levels. The formation of HIPs with THA and DOC was further verified by a shift in zeta potential values the more surfactant was bound to Exn. SEDDS formulation was developed and a droplet size of less than 30 nm with a homogenous distribution was obtained, and the physical stability of SEDDS formulations was proved over one week. The maximum payload of exenatide-THA and exenatide-DOC that could be dissolved in the lipophilic phase of the SEEDS was 0.54% and 0.17%, respectively. According to calculated log $D_{SEDDS/RM}$ in SIF and HBSS, the amount of Exn immediately released from Exn-DOC SEDDS is likely higher than that from Exn-THA SEDDS, showing the superiority of the cationic surfactant in protecting the peptide from the GI environment by forming more hydrophobic complexes. In vitro hemolysis studies revealed that the toxicity is concentration dependent, and both blank and loaded SEDDS displayed no significant hemolytic activity at a concentration of 0.25% (m/v). Compared to free Exn solution, Exn-DOC SEDDS and Exn-THA SEDDS exhibited a 3-fold and 10-fold increment in intestinal membrane permeability. Moreover, orally administered Exn-THA and Exn-DOC SEDDS resulted in a relative

bioavailability of 27.96 ± 5.24% and 16.29 ± 6.63%, respectively, confirming the comparatively higher potential of the cationic surfactant over the anionic surfactant.

6. New findings/Practical relevance of the work

- This is the first practical evidence of the theoretical extended version of QbD model for R&D which was successfully applied to identify the critical material attributes and process parameters that highly affect the quality of the final target nanosystem designed for oral GLP-1 analogue delivery.
- The novel biocompatible PLGA nanoparticles could successfully protect the encapsulated Lira from the enzymatic degradation in simulated GI on top of significantly enhancing the permeability across the Caco-2 cell intestinal model.
- Hydrophobic ion pairing of Exn with a cationic surfactant is investigated for the first time, and results proved the superiority of cationic surfactant THA over the model anionic surfactant DOC regarding the tested quality attributes.
- The novel biocompatible Exn-THA SEDDS hold high potential for oral delivery exceeding the relative bioavailability results obtained from preceding studies.
- Polymeric and lipid based NCs aiming to deliver Lira and Exn orally were successfully designed with characteristics complying with the predefined quality target product profile. The developed NCs could offer novel possibilities for oral GLP-1 analogues delivery to ensure effective management of T2DM.

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